(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) Publication number: **0 228 833**
A2

(12) **EUROPEAN PATENT APPLICATION**

(21) Application number: 86309526.1

(51) Int.Cl.⁴: **A 61 K 37/02**

(22) Date of filing: 08.12.86

(30) Priority: 24.12.85 JP 291471/85

(43) Date of publication of application:
15.07.87 Bulletin 87/29

(84) Designated Contracting States:
AT BE CH DE FR GB IT LI LU NL SE

(71) Applicant: **Takeda Chemical Industries, Ltd.**
27, Doshomachi 2-chome Higashi-ku
Osaka-shi Osaka, 541(JP)

(72) Inventor: **Ootsu, Koichiro**
9-26, Todaiji 3-chome Shimamoto-cho
Mishima-gun Osaka 618(JP)

(72) Inventor: **Goto, Giichi**
6-11, Kofudai 5-chome Toyono-cho
Toyono-gun Osaka 563-01(JP)

(74) Representative: **Lewin, John Harvey et al,**
Elkington and Fife High Holborn House 52/54 High Holborn
London WC1V 6SH(GB)

(54) **Immunostimulant agents.**

(57) A combined use of an IL-2-active substance with a muramyldipeptide exhibits a remarkably potent immunostimulant activity than the single use of the active ingredient.

EP 0 228 833 A2

Croydon Printing Company Ltd

0228833

IMMUNOSTIMULANT AGENTS

The present invention relates to an immunostimulant agent.

Attempts have been made in recent years, as immunostimulant agent and various viral infections, by using the so-called lymphokines such as interleukin-2 for immunopotentiation (J. Immunol., 125, 1904 (1980)). The above-mentioned interleukin-2, which is a macromolecular protein, has become producible in high purity and in large quantities and further at relatively low cost by making the best of genetic engineering techniques (Japanese Patent Laid-open No. 60-115328 which corresponds to EPC Publication No. 145390).

On the other hand, it is known that N-acetylmuramyl-L-alanyl-D-isoglutamine, which is included among the class of muramyldipeptides, is synthesized as a minimum structural unit necessary for the expression of bacterial cell wall adjuvant activity, and furthermore, various muramyldipeptides were synthesized. They exhibit potent adjuvant activity, typically antitumor activity or macrophage activation activity (Immunobiology and Immunotherapy of Cancer, edited by Yamamura et al., pp. 311-330, Elsevier/North Holland, New York, 1979).

Single application of the above-mentioned interleukin-2 (IL-2) or muramyldipeptide including as immunostimutant agents has been made but so far no fully satisfactory results have been obtained as yet.

Some means of enhancing the immunostimulant effect are known, for instance to increase the dose of the above-mentioned medicinal substances. However, high dosage treatment is difficult to practice due to manifestation of various adverse effects such as pyrexia, headache and exanthema.

In the course of their endeavors to develop a way of application of IL-2 as an immunostimulant agent, the present inventors found that the use of IL-2 in combination with a muramyldipeptide results in a remarkably enhanced immunostimulant activity, which can never be produced by single use of IL-2, and simultaneously can alleviate or prevent the above-mentioned adverse effects and the like. Further intensive study based on this finding has led to completion of the present invention.

The present invention is directed to:

(1). an immunostimulant agent which comprises a substance having interleukin-2 activity in combination with a muramyldipeptide and a pharmaceutically acceptable carrier;

(2). a method for immunostimulating a warm-blooded animal, which comprises administering a substance having interleukin-2 activity in combination with a muramyldipeptide to said animal; and

(3). a substance having interleukin-2 activity in combination with a muramyldipeptide, for use in the treatment of immunostimulating a warm-blooded animal.

The substance having interleukin-2 (IL-2) activity as mentioned above may be any substance having IL-2 activity, namely activity to allow indefinite propagation of T cells by passage with their functions being maintained.

Thus, for instance, mention may be made of natural IL-2 produced in animal bodies or in animal cells or genetically engineered IL-2, or a substance related thereto. The above-mentioned IL-2 or related substance, when it is a protein, may have or have not a sugar chain.

More specifically, it may be, for example, Polypeptide (A) [see EPC Publication No. 136200] which is produced using genetic engineering techniques and which

has the amino acid sequence in Fig. 1, and its fragments having a local amino acid sequence essential to its biological or immunological activity.

As the recombinant human IL-2, it is included a fragment lacking one amino acid from Polypeptide (A) at the amino terminus (EPC Patent Publication No. 91539), a fragment lacking four amino acids from Polypeptide (A) at the amino terminus (Japanese Patent Laid-open No. 126088/1985), and fragments lacking several amino acids from Polypeptide (A) at the carboxy terminus.

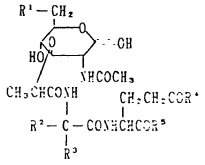
Furthermore, as the recombinant human IL-2, there are mentioned polypeptides produced by the elimination or substitution of other amino acids, as in the case of some constitutional amino acids in above-mentioned Polypeptide (A), e.g. a polypeptide produced by replacing the cysteine residue at the 125th position with a serine residue in Polypeptide (A) (Japanese Patent Laid-open No. 93093/1984 which corresponds to U.S. Patent No. 4,518,584).

The above-mentioned IL-2 may be chemically modified, for example with a polyethylene glycol derivative (e.g. Japanese Patent Laid-open No. 60-226821).

In the practice of the invention, human IL-2 which has the amino acid sequence shown in Fig. 1 is most preferably used. In that case, it may be a mixture of one further having a methionine residue (Met) at the amino terminus thereof and one having no such Met residue (Japanese Patent Laid-open No. 60-115528 which corresponds to EPC Publication No. 145390). The latter having no Met at the amino terminus but starting with alanine (Ala) (Japanese Patent Application No. 60-205873 which corresponds to EPC Publication No. 176299) is preferred.

As the substance having interleukin-2 activity, a recombinant non-glycosylated human interleukin-2 is preferred.

As the muramyldipeptide, there may be mentioned compounds of the formula (I)



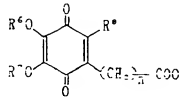
(I)

wherein R^1 is a hydroxyl group or a carboxylic acid residue, R^2 and R^3 each independently is hydrogen or a lower (C_{1-6}) alkyl group, which may optionally be substituted by a hydroxyl group, R^4 is a hydroxyl group or a lower (C_{1-6}) alkoxy group and R^5 is a hydroxyl group or a substituted or unsubstituted amino group, and physiologically acceptable salts thereof.

The compounds of formula (I) and salts thereof are all known compounds and are described in United States Patent No. 4,101,536, Japanese Patent Laid-open No. 54-63016, Japanese Patent Laid-open No. 54-79228 (which corresponds to EPC Publication No. 2677) and Japanese Patent Laid-open No. 55-111499 (which corresponds to U.S. Patent No. 4,369,178), for instance.

Thus, referring to the formula (I), the carboxylic acid residue represented by R^1 is, for example,

mycoloyl, stearoyl, oleoyl or a C_2-C_{50} carboxylic acid residue of the formula (II)



(II)

wherein R^6, R^7 and R^8 each independently is a lower (C_{1-4}) alkyl and n is an integer of 1 to 10, inclusive. Such carboxylic acid residue may further contain an intervening amino acid residue such as a glycine, alanine or ß-alanine residue. The lower alkyl group represented by R^2 and/or R^3 is preferably a C_1-C_4 alkyl, for example methyl, ethyl, propyl, isopropyl, butyl, isobutyl, sec-butyl or t-butyl. When substituted by a hydroxyl group, such lower alkyl is mentioned as hydroxymethyl or 2-hydroxyethyl, for instance.

The lower alkoxy represented by R^4 is preferably a C_1-C_3 alkoxy, namely methoxy, ethoxy, propoxy or isopropoxy. The hydroxyl group represented by R^4 may have a hapten-active substituent such as an ester residue of N-hydroxy-5-norbornene-2,3-dicarboxyimide.

The substituted or unsubstituted amino group represented by R^5 is a primary amino group or an amino group having one or two substituents. Examples of said substituents are lower (C_1-C_3) alkyl (e.g. methyl, ethyl, propyl, isopropyl, etc.), phenyl, and aralkyl (e.g. benzyl, phenethyl, etc.).

As the preferred embodiment of the muramyldipeptide, there may be mentioned the compound of the formula (I), in which R^1 is hydroxyl group or a C_2-C_{50} carboxylic acid residue of the formula (II) wherein R^6, R^7 and R^8 each independently is a lower (C_{1-4}) alkyl and n is an interger of 1 to 10, R^2 and R^3 is hydrogen or a lower (C_{1-6}) alkyl group which may optionally be substituted by a hydroxyl group, R^4 is a hydroxyl group or a lower (C_{1-6}) alkoxy group, and R^5 is a hydroxyl group or an amino group.

Typical examples of the muramyldipeptide of the above formula (I) are muramyldipeptide mycolic acid esters (e.g. 6-O-mycomycoloyl-N-acetylmuramyl-L-alanyl-D-isoglutamine, 6-O-mycomycoloyl-N-acetylmuramyl-L-seryl-D-isoglutamine, 6-O-nocardomycoloyl-N-acetylmuramyl-L-seryl-D-isoglutamine, 6-O-ursomycoloyl-N-acetylmuramyl-L-seryl-D-isoglutamine), muramyldipeptide fatty acid esters (e.g. 6-O-stearoyl-N-acetylmuramyl-L-alanyl-D-isoglutamine, 6-O-stearoyl-N-acetylmuramyl-L-seryl-D-isoglutamine, 6-O-oleoyl-N-acetylmuramyl-L-alanyl-D-isoglutamine), muramyldipeptide quinonylalkanoic acid esters (e.g. 6-O-[3-(2,3-dimethoxy-5-methyl-1,4-benzoquinon-6-yl)propionyl]-N-acetylmuramyl-L-valyl-D-isoglutamine, 6-O-[10-(2,3-dimethoxy-5-methyl-1,4-benzoquinon-6-yl)decanoyl]-N-acetylmuramyl-L-valyl-D-isoglutamine, 6-O-[10-(2,3-dimethoxy-5-methyl-1,4-benzoquinon-6-yl)decanoyl]-N-acetylmuramyl-L-seryl-D-isoglutamine), muramyldipeptides (e.g. N-acetylmuramyl-L-alanyl-D-isoglutamine (abbreviated as TMD-1)), N-acetylmuramylaminoisobutyryl-D-isoglutamine (abbreviated as TMD-5) and lower alkyl esters in the isoglutamine moiety of the above compounds (e.g. 6-O-[3-(2,3-dimethoxy-5-methyl-1,4-benzoquinon-6-yl)propionyl]-N-acetylmuramyl-L-valyl-D-isoglutamine methyl ester (abbreviated as TMD-76), 6-O-[10-(2,3-dimethoxy-5-methyl-1,4-benzoquinon-6-yl)decanoyl]-N-

acetylmuramyl-L-valyl-D-isoglutamine methyl ester (abbreviated as quinonyl-MDP-66), 6-O-[10-(2,3-dimethoxy-5-methyl-1,4-benzoquinon-6-yl)decanoyl]-N-acetylmuramyl-L-seryl-D-isoglutamine methyl ester.

In the practice of the present invention, TMD-1, TMD-5, TMD-76, quinonyl-MDP-66 and the like water-soluble muramylpeptides are particularly preferred among the above-mentioned muramyldipeptides.

The substance having IL-2 activity (IL-2-active substance) and the muramyldipeptide, which are to be used in accordance with the invention, have low toxicity, for example, the minimum lethal doses (MLDs) of IL-2 obtained by the manner of EPC Publication No. 145390 is not less than 10 mg/mouse (1 mg = 3.5×10^4 units/mg) (S.C.) and the minimum lethal doses (MLDs) for the muramyldipeptide is not less than 500 mg/kg (S.C.) in rats. Therefore, the substance having IL-2 activity and the muramyldipeptide can be used safely.

They are administered either orally or parenterally in doses dependent on the mode of use, purpose of use and other factors. The effective amount is desirably in a proportion of about 0.5 to 1,000 mcg, preferably about 50 to 400 mcg, of the muramyldipeptide per one mcg, as protein, of the IL-2-active substance (35 units (U) in terms of IL-2 activity; for the IL-2 activity assay, see Japanese Patent Laid-open No. 60-115528 which corresponds to EPC Publication No. 145390). The dose of the immunostimulant agent according to the present invention may vary also depending on the kind of IL-2 or muramyldipeptide employed. Generally, the effective amount of the daily dose for a warm-blooded mammals (e.g. mouse, cat, dog, cattle, sheep, goat, rabbit, human) as expressed in terms of IL-2 protein weight is preferably about 0.1 to

500 mcg/kg for mouse and about 0.001 to 10 mcg/kg for mammals other than mouse, more preferably 0.001 to 4 mcg/kg for mammals other than mouse in a form of injections, about 0.01 to 20 mcg/kg in a form of suppositories, about 0.001 to 2 mcg/kg in a form of drip infusion preparations, about 0.2 to 40 mcg/kg in a form of preparation for percutaneous absorption.

The immunostimulant agent according to the present invention which comprises an IL-2-active substance in combination with a muramyldipeptide can be made up for administration by mixing the substance or substances according to an appropriate known pharmaceutical process, using, as desired, one or more pharmaceutically acceptable carriers (including diluents, excipients and the like.) It is also possible to make up the respective substances into separate preparations or combine these active substances at the time of use into a single preparation containing them for administration by using a diluent, for instance. It is further possible to administer the above separate preparations to the same subject either simultaneously or at a certain time interval.

When preparing an agent for injection, as the carrier, there are mentioned distilled water, physiological saline and human serum albumin-suplemented distilled water or physiological saline.

As the carrier for an agent for suppositories, there are mentioned disaturated triglycerides, hydrogenated triglycerides, gelatin, glycerin, polyethylene glycol monostearate etc.

As the carrier for an agent for drip infusion preparations, there are mentioned distilled water, physiological saline, dextran sulfate solution.

As the carrier for an agent for percutaneous absorption, several kinds of ointment bases such as

glycerin, sodium lauryl sulfate, polyethylene glycol ointment, white wax etc. are usable.

The preparations of the present invention are made up in conventional manners employing the said carrier.

An example of the immunostimulant agent of the present invention, there are mentioned an antitumor agent for treatment of a warm-blooded animal carrying one or more tumors.

The antitumor agent is useful in the treatment or prevention of tumor in the warm-blooded mammal and produces remarkable effects in prolonging the lifespan in tumor-bearing mammals, for instance. As such target diseases, there may be mentioned various types of leukemia, malignant lymphoma, myeloma, malignant melanoma, malignant chorionic tumor, myoma, ovarian cancer, uterine cancer, prostatic cancer, spleen cancer, digestive organ cancer such as stomach cancer or intestinal cancer, lung cancer, esophageal cancer, cervical-cephalic cancer and cerebral tumor, among others.

The immunostimulant agent comprising an IL-2-active substance in combination with a muramyldipeptide in accordance with the present invention has potent immunostimulant activity which can never been exhibited by single use of each individual ingredient, and besides, it scarcely brings side effects.

Brief Description of the Drawing:

Fig. 1 shows an example of the amino acid sequence of IL-2 to be used in the practice of the present invention.

The following Examples are further illustrative of the working and mode of practice of the present invention but by no means limitative thereof.

The IL-2 employed in the following Examples 1 to 7 is a genetically engineered IL-2 species employing Escherichia coli DH1/pTF4 (IFO 14299, FERM BP-628) by

the manner described in Japanese Patent Laid-open No. 60-115528 (EPC Publication No. 145390).

The "Ala-species" of IL-2 employed in the following Examples 8 to 10 is a genetically engineered IL-2 species whose amino terminus amino acid is Ala-Pro-, which is obtained by the manner described in Example 5 of EPC Publication No. 176299 employing Escherichia coli N4830/pTB285 (IFO 14437, FERM BP-852).

Examples

Example 1

(Antitumor activity in the case of subcutaneous administration)

Meth-A fibrosarcoma cells (Meth-A tumor cells) (1×10^6 cells) were transplanted into each of female BALB/c mice weighing about 20 g subcutaneously in the flank using a syringe. Seven days after tumor transplantation, mice in which tumor was not smaller than a certain definite size were chosen and grouped and administration of the test agent was started. The agent was administered subcutaneously in the opposite flank relative to the tumor transplantation site once daily for 10 consecutive days. Each ingredient of the test agent was dissolved in physiological saline (solvent) supplemented in a form of a single preparation with 5% of normal mouse serum in a concentration such that the dose of the solution amounted to 0.1 ml/20 g of mouse body weight. The antitumor effect was evaluated by measuring the tumor weight in each mouse 21 days after tumor transplantation, determining the average tumour weight for each group and calculating the tumor weight ratio (T/C %) between the dosed group (T; 5 animals per group) and the untreated control group (C; 5 to 10 animals per group). The daily dose of each ingredients was expressed in terms of the ingredient weight (mcg) per mouse. The

results of single administration of IL-2 and those of administration of antitumor agents comprising IL-2 and N-acetylmuramyl-L-alanyl-D-isoglutamine (TMD-1) in accordance with the invention are shown in Table 1.

Table 1

Experiment No.	Dose (mcg/mouse/day) IL-2	TMD-1	Number of animals	Tumor weight (mg) Mean ± SD	Tumor weight ratio (T/C %)	Body weight gain (g) (day 7 to day 21)
I	Untreated control		9*	4,836± 996		2.5
	Solvent (control)		5	6,537± 595	135	3.6
	0	200	5	2,995± 846	62	1.3
	10	0	5	2,359±1,064	49	1.0
	1	200	5	2,548±1,037	53	0.9
	10	200	5	375± 504	9	-0.2
II	Untreated control		5	7,035±1,201		4.7
	Solvent (control)		5	5,717±2,060	81	3.0
	10	0	5	2,605± 709	37	1.4
	10	200	5	866± 622	12	0.4
	10	400	5	485± 479	7	0.5

*The test was started with 10 animals in this group but one animal died of tumor the day before autopsy.

Example 2

(Antitumor activity in the case of intravenous administration)

Meth-A tumor cells (1×10^6 cells) were transplanted into each of female BALB/c mice weighing about 20 g subcutaneously in the flank with a syringe. Seven days after tumor transplantation, mice in which tumor was not smaller than a certain definite size were chosen and

grouped and administration of the test agent was started. The agent was administered via the caudal vein once daily for 10 consecutive days. Each ingredient of the test agent was dissolved in physiological saline (solvent) in a form of a single preparation supplemented with 5% of normal mouse serum in a concentration such that the dose of the solution amounted to 0.2 ml/20 g of mouse body weight. The antitumor effect was evaluated by measuring the tumor weight in each animal 21 days after tumor transplantation, determining the average tumor weight for each group and calculating the tumor weight ratio (T/C%) between the dosed group (T; 5 animals per group) and the untreated control group (C; 10 animals). The results of single administration of IL-2 and those of administration of antitumor agents comprising IL-2 and N-acetylmuramyl-L-alanyl-D-isoglutamine (TMD-1) in accordance with the invention are shown in Table 2. The daily dose of each ingredient was expressed in terms of the ingredient weight (mcg) per mouse.

Table 2

Dose (mcg/mouse/day) IL-2	TMD-1	Number of animals	Tumor weight (mg) Mean ± SD	Tumor weight ratio (T/C %)	Body weight gain (g) (day 7 to day 21)
Untreated control		9*	6,890±1,150		4.3
Solvent (control)		5	5,315±1,439	77	3.7
10	0	5	4,368± 959	63	2.9
10	200	5	2,226±1,002	32	1.5
10	400	5	1,390±1,429	20	0.9

*The test was started with 10 animals in this group

but one animals died of tumor the day before autopsy.

Example 3

(Antitumor activity in the case of intravenous administration)

Under the same conditions as used in Example 2, an antitumor agent comprising IL-2 and 6-O-[10-(2,3-dimethoxy-5-methyl-1,4-benzoquinon-6-yl)decanoyl]-N-acetylmuramyl-L-valyl-D-isoglutamine methyl ester (quinonyl-MDP-66) was administered intravenously for 10 consecutive days. In this case, the antitumor effect was found to be as shown in Table 3.

Table 3

Dose (mcg/mouse/day) IL-2	Quinonyl-MDP-66	Number of animals	Tumor weight (mg) Mean ± SD	Tumor weight ratio (T/C %)	Body weight gain (g) (day 7 to day 21)
Untreated control		15	3,532±1,183		3.0
Solvent (control)		5	2,650± 363	75	1.7
10	0	5	1,999± 709	57	1.3
10	200	5	984± 693	28	0.3

Example 4

(Antitumor activity in the case of subcutaneous administration)

Under the same conditions as used in Example 1, antitumor agents comprising IL-2 and one of the two muramyldipeptides (TMD-5 or TMD-76) were administered subcutaneously for 10 consecutive days. The antitumor activity data thus obtained are shown in Table 4.

Table 4

Dose (mcg/mouse/day) IL-2	TMD-5*	TMD-76**	Number of animals	Tumor weight (mg) Mean ± SD	Tumor weight ratio (T/C %)	Body weight gain (g) (day 7 to day 21)
Untreated control			10	5,747± 741		3.3
10	0	0	5	3,072± 837	54	2.1
0	200	0	5	5,662±1,097	99	3.0
0	0	200	5	5,550±1,776	97	3.5
10	200	0	5	193± 171	3	-0.5
10	0	200	5	1,714± 868	30	0.3

*TMD-5: N-Acetylmuramylaminoisobutyryl-D-isoglutamine

**TMD-76: 6-O-[3-(2,3-dimethoxy-5-methyl-1,4-benzoquinon-6-yl)propionyl]-N-acetylmuramyl-L-valyl-D-isoglutamine

Example 5

(Injection preparation)

IL-2	10 mg
N-Acetylmuramyl-D-alanyl-L-isoglutamine	200 mg
Lactose	85 mg
HPC-L (hydroxypropylcellulose)	5 mg
Total	300 mg

The above four materials were mixed in the above proportions and then dissolved in distilled water (1000 ml) for injection or physiological saline and, following addition of human serum albumin (HSA) in a concentration of 0.5%, the resultant solution was filtered through a membrane filter (pore diameter: 0.22 µm). The filtrate thus obtained was distributed in 1-ml portions into

vials under aseptic conditions and lyophilized to give (1000 vials of) an antitumor preparation for injection. This injection preparation in each vial is to be dissolved in 1 ml of distilled water for injection at the time of use.

Example 6

(Injection preparation)

IL-2	100 mg
N-Acetylmuramylaminoisobutyryl-D-isoglutamine	100 mg
Total	200 mg

The above two ingredients were mixed together in the above proportions and dissolved in distilled water (1000 ml) for injection or physiological saline and, following addition of human serum albumin (HSA) in a concentration of 0.5%, the resultant solution was filtered through a membrane filter (pore diameter: 0.22um). The filtrate thus obtained was distributed in 1-ml portions into vials and lyophillized to give (1000 vials of) an antitumor preparation for injection. This injectable preparation in each vial is to be dissolved in 1 ml of distilled water for injection at the time of use.

Example 7

6-O-[10-(2,3-dimethoxy-5-methyl-1,4-benzoquinon-6-yl)decanoyl-N-acetylmuramyl-L-valyl-D-isoglutamine methyl ester (quinonyl-MDP-66) (2 g) was dispersed in 100 g of squalane and the dispersion was converted to a fine-particle dispersion in a Manton-Gaulin homogenizer. In the dispersion was dissolved 50 g of HCO-50 (Nikko Chemicals, Japan). After homogeneous dissolution, a 15-g portion was weighed and used as the oil phase.

Separately, 5.6 g of d-mannitol was dissolved in 100 ml of water and the solution was used as the water phase. The aqueous phase was added to the oil phase with stirring to make up and O/W emulsion. Further treatment in the Manton-Caulin homogenizer gave a fine-particle emulsion containing 200 mcg of the quinonyl compound per 1.2 ml. A vial was charged with 2.4 ml of this emulsion and 1 ml of an aqueous IL-2 solution having a concentration of 20 mcg/ml. After making the mixture homogeneous, the mixture was lyophilized to give an antitumor preparation. This injectable preparation is to be dissolved in distilled water for injection at the time of use.

Example 8

(Injection preparation)

IL-2 (Ala-species)	10 mg
N-Acetylmuramyl-D-alanyl-L-isoglutamine	200 mg
Lactose	85 mg
HPC-L (hydroxypropylcellulose)	5 mg
Total	300 mg

The above four materials were mixed in the above proportions and then dissolved in distilled water (1000 ml) for injection or physiological saline and, following addition of human serum albumin (HSA) in a concentration of 0.5%, the resultant solution was filtered through a membrane filter (pore diameter: 0.22 μm). The filtrate thus obtained was distributed in 1-ml portions into vials under aseptic conditions and lyophilized to give an antitumor preparation for injection. This injectable preparation in each vial is to be dissolved in 1 ml of distilled water for injection at the time of use.

Example 9

(Injection preparation)

IL-2 (Ala-species)	100 mg
N-Acetylmuramylaminoisobutyryl-D-isoglutamine	100 mg
Total	200 mg

The above two ingredients were mixed together in the above proportions and dissolved in distilled water (1000 ml) for injection or physiological saline and, following addition of human serum albumin (HSA) in a concentration of 0.5%, the resultant solution was filtered through a membrane filter (pore diameter: 0.22um). The filtrate thus obtained was distributed in 1-ml portions into vials and lyophillized to give 1000 vials of an antitumor preparation for injection. This injectable preparation in each vial is to be dissolved in 1 ml of distilled water for injection at the time of use.

Example 10

6-O-[10-(2,3-dimethoxy-5-methyl-1,4-benzoquinon-6-yl) decanoyl-N-acetylmuramyl-L-valyl-D-isoglutamine methyl ester (quinonyl-MDP-66) (2 g) was dispersed in 100 g of squalane and the dispersion was converted to a fine-particle dispersion in a Manton-Gaulin homogenizer. In the dispersion was dissolved 50 g of HCO-50 (Nikko Chemicals). After homogeneous dissolution, a 15-g portion was weighed and used as the oil phase. Separately, 5.6 g of d-mannitol was dissolved in 100 ml of water and the solution was used as the water phase. The aqueous phase was added to the oil phase with stirring to make up and O/W emulsion. Further treatment in the Manton-Gaulin homogenizer gave a fine-particle emulsion containing 200 mcg of the quinonyl compound per

1.2 ml. A vial was charged with 2.4 ml of this emulsion and 1 ml of an aqueous IL-2 (Ala-species) solution having a concentration of 20 mcg/ml. After making the mixture homogeneous, the mixture was lyophilized to give an antitumor preparation. This injectable preparation is to be dissolved in distilled water for injection at the time of use.

What we claim is:

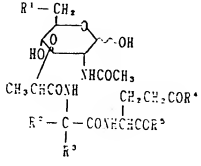
1. An immunostimulant agent which comprises a substance having interleukin-2 activity in combination with a muramyldipeptide and a pharmaceutically acceptable carrier.

2. An agent as claimed in Claim 1, wherein the agent is an anti-tumor agent.

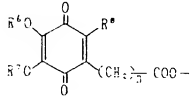
3. An agent as claimed in Claim 1 or 2, wherein the agent comprises a substance having interleukin-2 activity and a muramyldipeptide together with a pharmaceutically acceptable carrier.

4. An agent as claimed in any of claims 1 to 3, wherein the substance having interleukin -2 activity is a recombinant non-glycosylated human interleukin-2.

5. An agent as claimed in any of claims 1 to 4, wherein the muramyldipeptide is a compound of the formula:



wherein R^1 is hydroxyl group or a C_{2-50} carboxylic acid residue of the formula:



wherein R^6, R^7 and R^8 each independently is a C_{1-4} alkyl and n is an integer of 1 to 10, R^2 and R^3 is hydrogen or a C_{1-6} alkyl group which may optionally be substituted by a hydroxyl group, R^4 is a hydroxyl group or a C_{1-6} alkoxy group, and R^5 is a hydroxyl group or an amino group.

6. A substance having interleukin-2 activity in combination with a muramyldipeptide, for use in immunostimulating a warm-blooded animal.

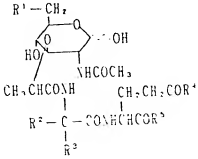
7. A use as claimed in Claim 6, which is a treatment of a warm-blooded animal carrying one or more tumors.

8. A use as claimed in claim 6 or 7, wherein the substance having interleukin-2 activity and the muramyldipeptide are used in a form of a single preparation.

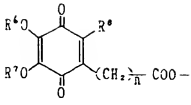
9. A use as claimed in any of claims 6 to 8, wherein the substance having interleukin-2 activity and the muramyldipeptide are separately used.

10. A use as claimed in any of claims 6 to 9, wherein the substance having interleukin-2 activity is a recombinant non-glycosylated human interleukin-2.

11. A use as claimed in any of claims 6 to 10, wherein the muramyldipeptide is a compound of the formula:



wherein R^1 is hydroxyl group or a C_{2-50} carboxylic acid residue of the formula:



wherein R^6, R^7 and R^8 each independently is a C_{1-4} alkyl and n is an integer of 1 to 10, R^2 and R^3 is hydrogen or a C_{1-6} alkyl group which may optionally be substituted by a hydroxyl group, R^4 is a hydroxyl group or a C_{1-6} alkoxy group, and R^5 is a hydroxyl group or an amino group.

Fig. 1

1
Ala Pro Thr Ser Ser Ser Thr Lys Lys Thr Gln Leu Gln

20
Leu Glu His Leu Leu Leu Asp Leu Gln Met Ile Leu Asn

Gly Ile Asn Asn Tyr Lys Asn Pro Lys Leu Thr Arg Met

40
Leu Thr Phe Lys Phe Tyr Met Pro Lys Lys Ala Thr Glu

60
Leu Lys His Leu Gln Cys Leu Glu Glu Glu Leu Lys Pro

Leu Glu Glu Val Leu Asn Leu Ala Gln Ser Lys Asn Phe

80
His Leu Arg Pro Arg Asp Leu Ile Ser Asn Ile Asn Val

100
Ile Val Leu Glu Leu Lys Gly Ser Glu Thr Thr Phe Met

Cys Glu Tyr Ala Asp Glu Thr Ala Thr Ile Val Glu Phe

120
Leu Asn Arg Trp Ile Thr Phe Cys Gln Ser Ile Ile Ser

133
Thr Leu Thr

European Patent Office

Application number:

86 309526.1 0228833

DECLARATION PURSUANT TO RULE 28, PARAGRAPH 4, OF THE EUROPEAN PATENT CONVENTION

The applicant has informed the European Patent Office that, until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, the availability of the micro-organism(s) identified below, referred to in paragraph 3 of Rule 28 of the European Patent Convention, shall be effected only by the issue of a sample to an expert.

IDENTIFICATION OF THE MICRO-ORGANISMS

Accession numbers of the deposits:

PERM BP - 628

PERM BP - 852